EP0585109

Publication Title:

N-Acetylglucosaminyl transferase, gene coding therefor, corresponding vectors and transformed hosts, processes for production thereof.

Abstract:

Abstract of EP0585109

A beta 1,6-N-acetylglucosaminyl transferase having the following properties: (1) Action: it transfers N-acetylglucosamine from UDP-N-acetylglucosamine to alpha -6-D-mannoside; (2) Substrate specificity: it shows a reactivity of about 79% for GnGnF-bi-PA, about 125% for GnGnGn-tri-PA and about 66% for GnM-Pa, when taking a reactivity for GnGn-bi-PA as 100%; (3) Optimum pH: 6.2 to 6.3; (4) Inhibition, Activation and Stability: Mn<2><+> is not necessary for expression of activity, and the activity is not inhibited in the presence of 20 mM EDTA; (5) Molecular weight: about 73,000 as determined by SDS-PAGE in the absence of reducing agent; and about 73,000 and about 60,000 as determined in the presence of a reducing agent; (6) Km value: 133 mu M and 3.5 mM for acceptor GnGn-bi-PA and donor UDP-GlcNAc, respectively; and (7) It includes the following peptide fragments: (1) Thr-Pro-Trp-Gly-Lys (2) Asn-Ile-Pro-Ser-Tyr-Val (3) Val-Leu-Asp-Ser-Phe-Gly-Thr-Glu-Pro-Glu-Phe-Asn-His-Ala-Asn-Tyr-Ala (4) Asp-Leu-Gln-Phe-Leu-Leu (5) Asn-Thr-Asp-Phe-Phe-Ile-Gly, and gene coding for said enzyme, and a process for production of the enzyme. Data supplied from the esp@cenet database - Worldwide

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number : **0 585 109 A2**

(12) EUROPEAN PATENT APPLICATION

(21) Application number : **93306718.3**

(51) Int. Cl.^{5} : **C12N 9/10**

(22) Date of filing : **24.08.93**

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(30) Priority : **24.08.92 JP 245950/92**
06.08.93 JP 237118/93

(43) Date of publication of application :
02.03.94 Bulletin 94/09

(84) Designated Contracting States :
AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL PT SE

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(54) N-Acetylglucosaminyl transferase, gene coding therefor, corresponding vectors and transformed hosts, processes for production thereof.

(57) A β1,6-N-acetylglucosaminyl transferase having the following properties :
(1) Action : it transfers N-acetylglucosamine from UDP-N-acetylglucosamine to α-6-D-mannoside ;
(2) Substrate specificity : it shows a reactivity of about 79% for GnGnF-bi-PA, about 125% for GnGnGn-tri-PA and about 66% for GnM-Pa, when taking a reactivity for GnGn-bi-PA as 100% ;
(3) Optimum pH : 6.2 to 6.3 ;
(4) Inhibition, Activation and Stability : Mn^{2+} is not necessary for expression of activity, and the activity is not inhibited in the presence of 20 mM EDTA ;
(5) Molecular weight : about 73,000 as determined by SDS-PAGE in the absence of reducing agent ; and about 73,000 and about 60,000 as determined in the presence of a reducing agent ;
(6) Km value : 133 μM and 3.5 mM for acceptor GnGn-bi-PA and donor UDP-GlcNAc, respectively ; and
(7) It includes the following peptide fragments :
(1) Thr-Pro-Trp-Gly-Lys
(2) Asn-Ile-Pro-Ser-Tyr-Val
(3) Val-Leu-Asp-Ser-Phe-Gly-Thr-Glu-Pro-Glu-Phe-Asn-His-Ala-Asn-Tyr-Ala
(4) Asp-Leu-Gln-Phe-Leu-Leu
(5) Asn-Thr-Asp-Phe-Phe-Ile-Gly,
and gene coding for said enzyme, and a process for production of the enzyme.

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The present invention relates to an enzyme which transfers N-acetylglucosamine from UDP-N-acetylglucosamine to α-6-D-mannoside (UDP-N-Acetylglucosamine: α-6-D-Mannoside β1-6N-Acetylglucosaminyl transferase; N-Acetylglucosaminyl-transferase; particularly as abbreviated "GnT-V" herein), a gene coding therefor, and a process for production thereof.

Asparagine type sugar chains found in glycoproteins are classified in three types, i.e., high mannose type, complex type and mixed type, on the basis of constituent sugars and the types of branching. Biosynthesis of these Asn type sugar chains starts with transfer of all sugar chain moiety of a lipid intermediate from the lipid intermediate to asparagine of a polypeptide chain during translation thereof in the lumina of the rough-surfaced endoplasmic reticulum.

After that glucose and a portion of mannose are removed in the rough-surfaced endoplasmic reticulum. However, a portion of glycoprotein having Asn type sugar chains located in the rough-surfaced endoplasmic reticulum remains there intact, and they retain with mannose type sugar chain. Other organella glycoproteins, cell surface glycoproteins or secretary glycoproteins are transferred to the Golgi body by Golgi transportation, and mannose is removed. In the Golgi body, Golgi body enzymes, N-acetylglucosaminyl transferases, introduce N-acetylglucosamine and branching structure is formed. By the formation of this branching structure, conversion of high mannose type sugar chain to mixed type sugar chain and complex sugar chain starts, and through the introduction of fucose and then of galactose in the trans Golgi region, and finally, introduction of sialic acid biosynthesis of Asn type sugar chain is completed.

In the steps of the formation of Asn-type sugar chains, it has been found that various enzymes catalyze the reaction. Among them, as enzymes catalyzing the transfer of N-acetylglucosamine, 6 kinds of N-acetylglucosaminyl transferases are known. Schachter et al., Brockausen, I. Caarver, J., and Schachter, H., Biochem. Cell. Biol., 66, 1134 (1988) named the six enzymes which transfer N-acetylglucosamine to the core structure of trimannosyl structure, Manαl-3(Manα1-6) Manβ1-4GlcNAcβ1-4G1cNAc as GnT-1 through GnT-VI.

Among them, the GnT-V is an enzyme participating in the formation of a β(1,6) branch structure (-[G-1cNAc-β(1,6)Man-α(1,6)Man]-). It is known that the β(1,6) branch structure increases in transformed cells and tumorigenic cells (Pierce, M., Arango. J., Tahir, S.H. and Hindsgunl, O., Biochem. Biophs. Res. Commun., 146, 679-684 (1987) and Arango, J. and Pierce, M., J. Cell. Biochem. 257, 13421-13427 (1982)).

Moreover, it was shown that the metastatic ability of tumorigenic cells corelates with the occurrence of β(1,6) branch (Hiraizumi, S., Takasaki, S., Shiroki, K., Kochibe, N., and Kobata, H., Arch. Biochem. Biophys. 280, 9-19 (1990)). In biopsies of human breast cancer, reportedly, in 50% of the cases the expression of the β(1,6) branch was accelerated (Dennis, J. W., and Laferrte, S. Cancer Res. 49, 945-950 (1989)).

In any case, it is known that the occurrence of β(1,6) branch structure is accompanied with an increase of GnT-V activity. As seen from the above, the GnT-V is not only important in that it catalyses formation of the β(1,6) branch structure in the biosynthesis of sugar chain, but also important in that it is related to easiness of the metastatic ability and the degree of malignancy of cancer cells.

Among these six N-acetylglucosaminyl transferases, human and rabbit cDNA structures for GnT-I were clarified (Kumar, R., Yang, J., Larsen, R.C., and Stanley, P. Procc. Natl. Acad. Sci. USA, 87, 9948-9952 (1990) and Sarkev, M. Hull, E., Nishikawa, Y., Simpson, R.J., Noritz, R.L., Dunn, R., and Schachter, H., Proc. Natl. Acad. Sci. U.S.A 88 234-238 (1991).

On the other hand, the presence of GnT-V was predicted from its enzyme activity, and its purification from various tissues was attempted, but it was very difficult (Nishikawa, A., Gu, J., Fujii, S., and Taniguchi, N. Biochem. Biophys. Acta 103, 313-318 (1990)). However, recently, isolation and purification of GnT-V from rat kidney was reported (Shoreibah, M., G., Handgaul, O., and Picerce, M., J. Biol. Chem. 267, 2920-2927 (1992)).

However, detailed information relating to the gene of said enzyme has not been obtained from rat GnT-V. For human GnT-V, any information including its enzymatic characteristics is not available. Not only GnT-V is very effective for diagnosis as a maker for degree of malignancy of cancer cells, but also it will be useful in that it will make it possible to design metastasis inhibitory agent by establishing a screening system for specific inhibitor. Therefore, it is desired to isolate and purify GnT-V of human origin, and to analyze the structures including the gene and to produce it in a large amount.

Accordingly, in one aspect, the present invention provides GnT-V of human origin. In addition, according to the present invention the gene for the present enzyme isolated, a process enabling large scale production of the enzyme using said gene is developed, and formation of homogeneous sugar chains in the production of a desired product by gene engineering becomes possible.

The present inventors noted a supernatant of cancer cells as a starting material for purification of the present enzyme. However, the present inventors also noted that purification of the enzyme was difficult in the use of a culture supernatant of cancer cells which can grow only in the presence of bovin serum or the like. Accordingly, the present inventors tried to condition various cancer cells for the growth in protein-free medium, and succeeded to establish tens of cancer cells conditioned to some decade protein-free media. Among them,

the present inventor detected an activity of the present enzyme in a culture supernatant from protein-free medium in which QG cells derived from human lung carcinoma (small cell cancer) were cultured, and succeeded to purify the present enzyme.

Accordingly, in one aspect we provide a β1,6-N-acetylglucosaminyl transferase determined by us as having the following properties:

(1) Action: it transfers N-acetylglucosamine from UDP-N-acetylglucosamine to α-6-D-mannoside;
(2) Substrate specificity: it shows a reactivity of about 78% for GnGnF-bi-PA, about 125% for GnGnGn-tri-PA and about 66% for GnM-PA, when taking a reactivity for GnGn-bi-PA acceptor as 100%;
(3) Optimum pH: 6.2 to 6.3;
(4) Inhibition, Activation and Stability: Mn^{2+} is not necessary for expression of activity, and the activity is not inhibited in the presence of 20 mM EDTA;
(5) Molecular weight: about 73,000 as determined by SDS-PAGE in the absence of reducing agent; and about 73,000 and about 60,000 as determined in the presence of a reducing agent;
(6) Km value: 133 μM and 3.5 mM for acceptor GnGn-bi-PA and donor UDP-GlcNAc, respectively; and
(7) It includes the following peptide fragments:
(1) Thr-Pro-Trp-Gly-Lys
(2) Asn-Ile-Pro-Ser-Tyr-Val
(3) Val-Leu-Asp-Ser-Phe-Gly-Thr-Glu-Pro-Glu-Phe-Asn-His-Ala-Asn-Tyr-Ala
(4) Asp-Leu-Gln-Phe-Leu-Leu
(5) Asn-Thr-Asp-Phe-Phe-Ile-Gly

Alternatively stated, the present β1,6-N-acetylglucosaminyl transferase has an amino acid sequence comprising the amino acid sequence shown in SEQ ID No: 8, or an amino acid sequence wherein one or more than one amino acid residue is modified in the amino acid sequence shown in SEQ ID No: 8. Herein, the amino acid modification means that one or more amino acid residues are added, deleted and/or replaced with other amino acids, but the enzyme activity is available.

The present invention further provides a process for production of the above-mentioned enzyme comprising the steps of culturing QG cells derived from human lung carcinoma, and recovering said enzyme from the culture.

The present invention still further provides a process for production of the above-mentioned enzyme comprising the steps of culturing or raising a host transformed with a DNA coding for said enzyme, and recovering the enzyme from the culture or raised host.

The present invention also relates to gene system encoding the enzyme i.e., DNA, expression vectors and transformed host.

BRIEF EXPLANATION OF THE DRAWINGS

Figure 1 shows an elution profile of Phenyl-Sepharose column chromatography, wherein solid circles linked with solid lines show GnT-V activity. The elution was carried out by decreasing concentration of ammonium sulfate from 40% to 0%. Concentration of ammonium sulfate is shown by a broken line.

Fig. 2 shows an elution profile of hydroxyapatite column chromatography, wherein open circles linked with solid lines show elution profile of protein, and solid circles linked with solid lines show GnT-V activity. In this figure, the arrow shows the starting point of flow of elution buffer. The elution was carried out by forming a concentration gradient from 50 mM to 300 mM phosphate buffer. Concentration of phosphate buffer is shown by a broken line.

Fig. 3 shows an elution profile of UDP-hexanolamine Agarose-column chromatography, wherein open circles linked with solid lines show an elution profile of protein, and solid circles linked with solid lines show GnT-V activity. In the figure, the arrow shows the start point of flow of elution buffer. The elution was carried out by flowing 0.3 M NaCl at a flow rate of 3 ml/hour.

Fig. 4 shows an elution profile of GnGn-Asn Sepharose-column chromatography, wherein open circles linked with solid lines show an elution profile of protein, and solid circles linked with solid lines show GnT-V activity. In the figure, the arrow shows the start point of flow of elution buffer. The elution was carried out by flowing 0.3 M NaCl at a flow rate of 3 ml/hour.

Fig. 5 shows a result of SDS polyacrylamide gel electrophoresis of the enzyme.

Fig. 6 shows a result of native polyacrylamide gel electrophoresis of the enzyme.

Fig. 7 shows a peptide mapping analysis of the enzyme.

Fig. 8 is a graph showing optimum pH of the enzyme.

Fig. 9 represents a construction process of an expression vector.

Fig. 10 is an elution profile of a high performance liquid chromatography showing that the expression prod-

uct, GnT-V, converts a substrate GnGn-bi-PA to a product GnGnGn-tri'-PA.

SPECIFIC EMBODIMENT

QG cells derived from human lung carcinoma cells (small cell cancer) can be cultured by any method conventionally used for culturing animal cells. The QG cells can be cultured by static culture in a protein free medium, that is, the medium is not supplemented with any extrageneous proteins. Note, a culture of the QG cells derived from human lung carcinoma (small cell cancer) was designated as Human lung carcinoma SBM331 and deposited with Fermentation Research Institute Agency of Industrial Science and Technology, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305, Japan, as FERM BP-3967 on August 18, 1992, under Budapest Treaty.

Enzyme activity can be easily assayed by labeling an acceptor with a radioisotope or with fluorescent substance, adding the enzyme and separating the reaction product by high performance liquid chromatography (HPLC). More specifically, the method reported by Taniguchi et al. (Taniguchi, N., Nishikawa, A., Fujii, S., and Gu. J., Methods Enzymol. 179, 397-408 (1989); Nishikawa, H., Gu, J., Fujii, S., and Taniguchi, N., Biochem. Biophys. Acta, 1035, 313-318 (1990)) may be followed.

Namely, the enzyme activity of GnT-V can be assayed using GnGn-2-aminopyridine (GnGn-bi-PA) as an acceptor, and UDP-GluNAc (Sigma) as a donor. Activity is shown by an amount of transferred N-acetylglucosamine/hour/mg protein. Note, an amount of protein can be measured according to a reported method (Redinbaugh, M.G., and Turley, R.B., Anal. Biochem. 153, 267-271 (1986)) using a BCA kit (Pierce Chemical Company, Rockford, IL) and using bovine serum albumin as a standard.

The present enzyme may be isolated and purified by using procedures singly or in combination conventionally used for purification of a protein. In addition, as a useful purification method for the present enzyme, an affinity of the enzyme to an acceptor or donor may be used. According to one embodiment of the present invention, a culture supernatant from a protein-free culture is first concentrated by ultrafiltration, and an active fraction is obtained by hydrophobic chromatography using Phenyl-Sepharose.

Next, after absorption to Hydroxylapatite, an active fraction is eluted, and the active fraction is subjected to acceptor column chromatography (UDP-hexanolamine-Agarose) and donor column chromatography (GnGn-Asn-Sepharose), followed by sodium dodecyl sulfatepolyacrylamide gel electrophoresis in the absence of reducing agent to obtain a polypeptide showing a single band.

A particular example for the purification is described in Example 2. As seen from Example 2, the present inventors succeeded to isolate and purify a 20,000-fold purified GnT-V by 4-step purification procedure from the above-mentioned culture supernatant concentrate of protein-free culture, and determined partial amino acid sequences of the enzyme. The enzyme was obtained for the first time by a particular combination of procedures used for isolation and purification of a protein, under particular conditions.

The enzyme having been isolated and purified, and structural properties of the enzyme clarified by us as described hereinafter, the enzyme can be isolated and purified using the known properties as an indicator according to any other combination of conventional procedures used for isolation and purification of a protein. Moreover, synthetic probes can be prepared on the basis of partial amino acid sequences of the present enzyme disclosed herein, genomic library and cDNA library derived from avian, amphibia, mammal and the like can be screened with said probes to isolate genes, and the gene can be used to produce a corresponding enzyme.

An enzyme isolated and purified as described herein has the following properties.

1. Action: it transfers N-acetylglucosamine from UDP-N-acetylglucosamine to α-6-D-mannoside;
2. Substrate specificity: it shows a reactivity of about 78% for GnGnF-bi-PA, about 125% for GmGmGm-tri-PA, and about 66% for GnM-PA, when taking a reactivity for GnGn-bi-PA acceptor as 100%;
3. Optimum pH for reaction: 6.2 to 6.3;
4. Inhibition, Activation and Stability: Mn^{2+} is not necessary for expression of the activity, and the activity is not inhibited in the presence of 20 mM EDTA;
5. Molecular weight: as a result obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), it shows a single band corresponding to a molecular weight of about 73,000 under a non-reducing condition, and it shows a band corresponding to a lower molecular weight of about 60,000 in addition to the above-mentioned band of about 73,000. In addition, when the isolated and purified enzyme is subjected to native polyacrylamide gel electrophoresis, the extraction samples from the sections of the native polyacrylamide gel exhibit the enzyme activity in a density dependent manner. From the above result, the isolated and purified protein is confirmed to be the desired GnT-V. A particular result is described in Example 3.

On the other hand, two bands in an SDS-polyacrylamide gel electrophoresis under the reducing con-

dition are excised from the gel, and each fraction is digested with trypsin followed by reverse phase high performance liquid chromatography and peptide mapping. As a result, both the 60 kDa fraction and 73 kDa fractions show similar elution paterns. From this result, it is concluded that both the proteins are derived from the same protein even though there is possibility that one of or both the enzymes have been enzymatically cleaved;

6. Km value: 133 μM and 3.5 mM for acceptor GG-bi-PA and donor UDP-GlcNAc, respectively; and

7. Peptide fragments: amino acid sequences of peptide fragments obtained by digestion of the enzyme with trypsin were as follows:

(1) Thr-Pro-Trp-Gly-Lys
(2) Asn-Ile-Pro-Ser-Tyr-Val
(3) Val-Leu-Asp-Ser-Phe-Gly-Thr-Glu-Pro-Glu-Phe-Asn-His-Ala-Asn-Tyr-Ala
(4) Asp-Leu-Gln-Phe-Leu-Leu
(5) Asn-Thr-Asp-Phe-Phe-Ile-Gly

Further a gene coding for the enzyme may be obtained by preparing oligonucleotide probes on the basis of the amino acid sequences of the peptide fragments and screening a genomic library or a cDNA library using the probes.

Gene of the present invention may be cDNA, genomic DNA or chemically synthesized DNA. For example, cDNA can be cloned by polymerase chain reaction (PCR) using nucleotide primers designed on the basis of the partial amino acid sequences, as shown in Example 5, of GnT-V purified from human cells such as QG cells derived from human lung carcinoma. A particular embodiment of the cloning is shown in Example 8.

The invention further provides DNA which codes for a protein having GnT-V activity and which hybridizes with the nucleotide sequence shown in Example 8.

A nucleotide sequence of DNA coding for the present GnT-V and cloned in Example 8, and an amino acid sequence predicted from the nucleotide sequence are shown in SEQ ID No: 8.

Thus, once an amino acid sequence is determined, various modified GnT-V, such as polypeptide wherein one or more amino acids are added to the above-mentioned native amino acid sequence and still maintaining GnT-V activity, a polypeptide wherein one or more amino acids are deleted from the native amino acid sequence and still maintaining GnT-V activity, a polypeptide wherein one or more amino acids are replaced with other amino acids in the native amino acid sequence and still maintaining GnT-V activity, and a polypeptide including any combination of the above-mentioned amino acid addition modification, amino acid deletion modification and amino acid replacement modification, can be designed and produced.

Although the number of amino acids in the above-mentioned amino acid addition, deletion or replacement modification is not limited to the particular number, for example, for the addition modification, the number depends on the purposes of the modification, for example, depends on the number of amino acids of a functional protein used for formation of a hybride protein with the present GnT-V (for example, known proteins for purification or stabilization such as maltose-binding protein, or various biologically active proteins for example cytokines such as IL-3, IL-11), or signal peptide attached to the present protein. For example, the number of added amino acids are 1 to 30, preferably 1 to 10.

For the deletion modification, the number of deleted amino acids is designed and determined so that the modified protein maintains GnT-V activity, and is for example 1 to 30, preferably 1 to 20, or the number of amino acids in regions other than an active region. For the replacement modification, the number of replaced amino acids is designed and determined so that the modified protein maintains GnT-V activity, and is for example 1 to 10, preferably 1 to 5.

Although the nucleotide sequence is shown in SEQ ID No: 8 as a nucleotide sequence coding for GnT-V, gene of the present invention coding for GnT-V is not limited to it. Once an amino acid sequence of native GnT-V or an amino acid sequence of modified GnT-V is determined, various nucleotide sequences coding for the same amino acid sequence by different codons on the basis of the degeneracy of genetic codons can be designed and prepared. In this case, codons used with a high frequency in a host chosen are preferably used.

Although cDNA can be obtained as a gene coding for the native GnT-V of the present invention, as described in Example 8, but not limited thereto. Namely, once a nucleotide sequence coding for an amino acid sequence of the native GnT-V is determined, a gene coding for the native GnT-V can be cloned by a strategy different from the strategy specifically described herein; and also, can be cloned from the genome of cells producing the native GnT-V.

Where a gene is cloned from the genome, various primer nucleotides or probe nucleotides used in Example 8 may be used as probes for screening genomic DNA fragments. Moreover, other probes designed on the basis of the nucleotide sequence shown in SEQ ID No: 8 may be used. General procedure for cloning a desired DNA from genome is well known in the art (Current Protocols in Molecular Biology, John Wiley and Sons, Chapters 5 and 6).

In addition, a gene coding for the native enzyme may be prepared by chemical synthesis. Chemical synthesis of DNA can be easily carried out using an automated DNA synthesizer conventionally used in the art, such as a 396 DNA/RNA synthesizer (Applied Biosystems). Accordingly, a person with ordinary skill in the art can easily synthesize the nucleotide sequence shown in SEQ ID No: 8.

A gene coding for the native enzyme using codons different from native codons can be chemically synthesized as described above, and further can be obtained by a conventional procedure such as site-directed mutagenesis using a DNA or RNA having the nucleotide sequence shown in SEQ ID No: 8 as a template in combination with a mutagenic primer (see, for example, Current Protocols In Molecular Biology, John Wiley & Sons, Chapter 8).

Once a suitable gene coding for the enzyme has been cloned, the gene can be used to produce a recombinant enzyme e.g. according to a conventional gene recombination technique. Namely, a DNA encoding a GnT-V of the present invention is inserted into a suitable vector, and the vector is introduced into an adequate host, and the host is cultured or raised, and the GnT-V is recovered from the culture (cells or medium) or the raised host.

As a host, prokaryote or eukaryote can be used. As prokaryote, bacteria, especially Escherichia coli, Bacillus, such as B. subfilis may be used. As eukaryote, eukaryotic microorganisms, for example, yeast such as the genus saccharomyces, such as S. cerevisiae, insect cells such as cells of Spodoptera frugiperda, cells of Trichoplusia ni or cells of Bombyx mori, animal cells such as human cells, monkey cells, mouse cells can be used. An insect per se., such as Bombyx mori, Trichoplusia ni, or the like can be used.

As expression vectors, plasmid, phage, phagemid, virus (baculovirus for insect, vaccinia virus for animal cells) and the like can be used. A promoter in an expression vector is selected depending on host. For example, as bacterial promoters, for example, lac promoter, trp promoter and the like are used; and as yeast promoters, for example, adhl promoter, pqk promoter and the like are used. As promoters for insect, for example, baculovirus polyhedrin promoter and the like can be used, and promoters for animal cells, for example, early or late promoter for Simian virus 40 and the like may be used.

Transformation of a host with an expression vector can be carried out according to conventional procedures well known in the art, and these procedure are described in Current Protocols in Molecular Biology, John Wiley & Sons. Transformants can be cultured according to conventional procedures.

Purification of GnT-V from a culture can be carried out according to a conventional procedure for isolation and purification of a protein, for example, using ultrafiltration, various column chromatography such as Sepharose chromatography.

EXAMPLES

Next, the present invention is further explained by but not limited to the following examples.

Example 1. Culturing of GnT-V producing cells and preparation of culture supernatant

QG cells (FFRM BP-3967) derived from human lung carcinoma (small cell cancer) were cultured in an opticell 5300 (Charles River Inc. Wilmington, MA). 2×10^9 cells were inoculated to a porous ceramic growing chamber (effective surface > 32,000 cm^2), and grown to a confluent state using Ham's F-12 medium (Flow) containing 5% bovine serum (GIBCO).

Next, protein-free Ham's F-12 medium containing 10^{-8}M sodium selenite was continuously added to gradually decrease an amount of serum. After culturing for 2 weeks, 140L of a culture supernatant was obtained. This culture supernatant was concentrated by about 100 folds using an ultrafiltration apparatus equipped with a UF-membrane (Milipore, Bedford, MA). 1.4L of the concentrated supernatant was used as a starting material for purification.

Example 2. Purification of enzyme from the concentrated culture supernatant

(1) Preparation of UDP-hexanolamine agarose and GlcNAcβ1-2Manα1-3-(GlcNAcβ1-2Manα1-6) Manβ1-4GlcNAcβ1-4GlcNAc-asparagine (GnGn-Asn) Sepharose

UDP-hexanolamine Agarose was purchased from Sigma (St. Louis, MO). GnGn-Asn was prepared from human transferrin. First, transferrin was digested twice with Pronase (Boehringer Mannheim) in 0.1M borate buffer (pH 8.0) at 37°C for 12 hours, and resulting sugar peptides were separated by a gel filtration column (Toyoperl HW-40, 2.6 × 100 cm, Tosoh, Tokyo) previously equilibrated with 10 mM acetate buffer (pH 6.0).

Next, the separated sugar peptide was digested with enzymes sialidase (from Arthrobactor ureafa-

cience, available from Nakalai Tesque, Kyoto, Japan) and β-galactosidase (from jack beam, available from Seikagaku Co., Japan) to obtain GnGn-Asn. GnGn-Ans Sepharose was obtained by reacting 100 μmol GnGn-Asn with 10 ml of activated CH-Sepharose (Pharmacia) to linking them.

(2) Phenyl-Sepharose column chromatography 280 ml of Phenyl-Sepharose was filled to a 4.5 × 18 cm column, and the column was equilibrated with 1.5L of 20 mM potassium phosphate buffer (pH 6.8, ammonium sulfate 40% saturation). 1.3L of the concentrated culture supernatant prepared in Example 1 was saturated with ammonium sulfate to 40% saturation, centrifuged at 3000 × G for 30 minutes, and the resulting supernatant was absorbed to the column.

Elution was carried out by a linear gradient from ammonium sulfate 40% to 0% in 20 mM potassium phosphate buffer (pH 6.8); at a flow rate of 3 ml/min. Absorption at 280 nm and GnT-V enzyme activity were measured. As shown in Fig. 1, the peak of GnT-V was eluted after the peak of absorption at 280 nm.

(3) Hydroxyapatite column chromatography

The fractions showing GnT-V enzyme activity eluted from the Phenyl-Sepharose column chromatography were collected, and concentrated with an Amicon YM-30 membrane while simultaneously exchanging the original medium with 20 mM potassium phosphate (pH 6.8). The concentrated fraction was then adsorbed to 55 ml of hydroxyapatite equilibrated with 300 ml of 20 mM potassium phosphate buffer (pH 6.8). Elution was carried out by a linear gradient of 50 mM - 300 mM potassium phosphate buffer (pH 6.8) at a flow rate of 3 ml/min. An elution profile of the hydroxyapatite chromatography is shown in Fig. 2. The peak of GnT-V enzyme activity was eluted separately from most of other proteins.

(4) UDP-hexanolamine Agarose-column chromatography

Next, the fractions from the hydroxyapatite column were collected, and the original medium was exchanged with 10 mM potassium phosphate buffer (pH 6.25) using an Amicon YM-30 membrane. The resulting fraction was adsorbed to 20 ml of UDP-hexanolamine Agarose column previously equilibrated with 100 ml of 10 mM potassium sulfate buffer (pH 6.25). Enzyme was eluted with 10 mM potassium phosphate containing 0.3M NaCl, at a flow rate of 15 ml/min. This elution profile is shown in Fig. 3.

(5) GnGn-Asn Sepharose column chromatography

Finally, GnGn-Asn Sepharose column chromatography was carried out. The active fraction from the UDP-hexanolamine Agarose column was adsorbed to 4 ml of GnGn-Asn Sepharose equilibrated with 20 ml of 10 mM potassium phosphate buffer (pH 6.25) containing 0.3M NaCl, and after washing the column with same buffer, elution was carried out with 30 mM Tris-HCl (pH 9.0) containing 0.3M NaCl at a flow rate of 3 ml/hour. A result is shown in Fig. 4.

Purification yield of the above-mentioned purification process is shown in Table 1. Each step provided more than 70% yield, and final purification ratio was about 20,000.

Table 1

Purification steps from culture supernatant of QG cells derived from human lung carcinoma (small cell cancer), cultured in protein-free medium, and corresponding purification yield

Steps	Protein (mg)	Specific Activity (nmol/h/mg)	Yield %	Purification ratio
Medium[(a)]	3,250	0.4	100	1
Phenyl-Sepharose	330	2.9	71	7
Hydroxyapatite	110	8.4	67	20
UDP-hexanolamine-Agarose	12.6	56.7	52	135
GnGn-Asn-Sepharose	0.06	8323	37	19,800

(a) 1.3L culture supernatant

Example 3. SDS polyacrylamide gel electrophoresis and native polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was carried out according a reported method (Laemmli, U.K., Nature 227, 680-685, 1970), using 10 - 15% gradient polyacrylamide gel under the reducing condition and non reducing condition. As molecular weight makers, α-lactalbumin (Mmolecular weight 14,400), soybean trypsin inhibitor (M.W. 20,100), carbonic anhydrase (M.W. 30,000), ovalbumin (M.W. 43,000), bovine serum albumin (M.W. 67,000), and phosphorylase b(M.W. 94,000) were used.

A photograph of the gel prepared by staining the present enzyme with Coomassie Brilliant Blue is shown in Fig. 5. In an SDS polyacrylamide gel electrophoresis under the non-reducing condition, a single band having a molecular weight of 73 kDa as determined by comparing with the above-mentioned molecular weight makers was observed. On the other hand in an SDS polyacrylamide gel electrophoresis under the reducing condition, a band of 60 kDa in addition to the band of 73 kDa were observed on the gel. A photograph of a gel of native polyacrylamide gel electrophoresis stained with Coomassie Brilliant Blue and enzyme activity of sections excised from the native polyacrylamide gel electrophoresis are shown in Fig. 6. Levels of the enzyme activity were observed dependent on the densities of the bands. From the above, it was confirmed that the isolated and purified protein is a desired GnT-V.

Example 4. Peptide mapping analysis

SDS polyacrylamide gel electrophoresis was carried out under a reducing condition, and bands corresponding to 60 kDa and 73 kDa were excised from the gel. To the sliced gel pieces was added 30 mM Tris-HCl (pH 6.5) containing 0.5% SDS, which was then boiled, and protein was extracted with an elctroelution apparatus. After the elution, the protein was digested with 200 ng of trypsin (Sigma, St. Louis, Mo), dissolved in 50 mM Tris-HCl (pH 8.0) at 37°C for 11 hours.

The digestion product was applied to a reverse phase high performance liquid chromatography (Chemcosorb 3 ODS-H, Osaka Japan, 2.1 × 75 mm), and eluted under an acidic condition (0.1% TFA) using 0 - 60% acetonitrile gradient. An elution profile is shown in Fig. 7. The proteins of 60 kDa and 73 kDa show similar elution profiles and therefore it is concluded that both the proteins comprise basically same polypeptide, though there is possibility that they are partially enzymatically digested.

Example 5. Determination of partial amino acid sequences

To determine the amino acid sequences, the peptide fragments obtained in Example 2 from purified GnT-V were subjected to a Gas-phase sequencer (Applied Biosystem Inc. Foster City, CA), and the following sequences were obtained.

(1) Thr-Pro-Trp-Gly-Lys
(2) Asn-Ile-Pro-Ser-Tyr-Val
(3) Val-Leu-Asp-Ser-Phe-Gly-Thr-Glu-Pro-Glu-Phe-Asn-His-Ala-Asn-Tyr-Ala
(4) Asp-Leu-Gln-Phe-Leu-Leu
(5) Asn-Thr-Asp-Phe-Phe-Ile-Gly

Example 6. Substrate Specificity

Substrate specificity was determined using the following compound as sugar acceptors according to the above-mentioned enzyme activity measuring method.

```
                        GlcNAcβ1 → 2Manα1 ↘
                                            6
GnGn-bi-PA                                   Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA
                                            3
                        GlcNAcβ1 → 2Manα1 ↗

                        GlcNAcβ1 → 2Manα1 ↘                 Fucα1 ↘
                                            6                       6
GnGnF-bi-PA                                  Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA
                                            3
                        GlcNAcβ1 → 2Manα1 ↗

                        GlcNAcβ1 → 2Manα1 ↘                 Fucα1 ↘
                                            6                       6
GnGF-bi-PA                                   Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA
                                            3
       Galβ1 → 4GlcNAcβ1 → 2Manα1 ↗

       Galβ1 → 4GlcNAcβ1 → 2Manα1 ↘                 Fucα1 ↘
                                            6                       6
GGnF-bi-PA                                   Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA
                                            3
                        GlcNAcβ1 → 2Manα1 ↗

                        GlcNAcβ1 → 2Manα1 ↘
                                            6
Gn(Gn)Gn-bi-PA            GlcNAβ1 → 4 Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA
                                            3
                        GlcNAcβ1 → 2Manα1 ↗

                        GlcNAcβ1 → 2Manα1 ↘                 Fucα1 ↘
                                            6                       6
Gn(Gn)GnF-bi-PA          GlcNAcβ1 → 4 Manβ1 → 4GlcNAcβ1 → 4GlcNAc-PA
                                            3
                        GlcNAcβ1 → 2Manα1 ↗
```

```
            Galß1 → 4GlcNAcß1 → 2Manα1 ↘
                                          6
    GG-bi-PA                                Manß1 → 4GlcNAcß1 → 4GlcNA
                                          3
            Galß1 → 4GlcNAcß1 → 2Manα1 ↗
```

```
                          Manα1 ↘
                                  6
    MM-PA                           Manß1 → 4GlcNAcß1 → 4GlcNAc-PA
                                  3
                          Manα1 ↗
```

```
                    GlcNAcß1 → 2Manα1 ↘
                                        6
    GnM-PA                                Manß1 → 4GlcNAcß1 → 4GlcNAc-PA
                                        3
                                Manα1 ↗
```

```
                    GlcNAcß1 → 2Manα1 ↘
                                        6
    GnGnGn-tri-PA                         Manß1 → 4GlcNAcß1 → 4GlcNAc-PA
                                        3
                    GlcNAcß1 → 2Manα1 ↗
                               4
                    GlcNAcß1 ↗
```

Lacto-N-triaose-PA GlcNAcβ1 → 3Galβ1 → 4Glc-PA

Lacto-N-neotetraose-PA Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc-PA

```
                    GlcNAcß1 ↘
                               6
                    GlcNAcß1 → 2Manα1 ↘
                                        6
    GnGnGn-tri'-PA                        Manß1 → 4GlcNAcß1 → 4GlcNAc-PA
                                        3
                    GlcNAcß1 → 2Manα1 ↗
```

GnGnF-, GnGF-, GGnF-, Gn(Gn)GnF-, Gn(Gn)Gn-, GG-, GnGn-, MM- and GnM- were isolated from the bovine IgG treated with anhydrous hydrazine and then labeled with a fluorescence label according to Hase et al. method (Hase, S., Ibuki, T., and Ikehara, T., J. Biochem. 95, 197-203 (1984). The purified GnT-V was diluted with PBS buffer to a final concentration of 0.1 mg protein/ml, and then reacted with each fluorescence-labeled sugar acceptor. A result is shown in Table 2.

Table 2

Substrate Specificity of the Present Enzyme to Various Sugar Acceptors	
Acceptor (70 μM)	Relative reaction rate
GnGn-bi-PA	100
GnGnF-bi-PA	78
GnGF-bi-PA	3
GGnF-bi-PA	0.2
Gn(Gn)Gn-bi-PA	0
Gn(Gn)GnF-bi-PA	0
GG-bi-PA	0
MM-PA	0
GnM-PA	66
GnGnGn-tri-PA	125
Lacto-N-triaose-PA	0
Lacto-N-neotetraose-PA	0

It was found that the present enzyme reacts with all of GnGnF-bi-PA, GnM-PA and GnGnGn-tri-PA.

Example 7. Optimum pH

Assay mixtures containing 0.2M GlcNAc, 40 mM EDTA, 1% Triton X-100, and 40 mM UDP-GlcNAc in 0.25M potassium phosphate buffer of pH 6.0, 6.25, 6.5 or 7.0. To 25 μl of the assay mixture were added 15 μl of a test sample and 10 μl of a substrate (GnGn-bi-PA), and the mixture was incubated at 37°C for 4 hours. A result is shown in Fig. 8.

Example 8. Isolation and identification of cDNA for GnT-V and Construction of expression vector

Oligomer S18 shown in SEQ ID No: 6 was synthesized on the basis of the amino acid sequence shown in SEQ ID No: 3 obtained in Example 5, and oligomer A21 shown in SEQ ID No: 7 was synthesized on the basis of the amino acid sequence shown in SEQ ID No: 5.

Double-stranded cDNA was synthesized from RNA having poly A prepared from QG cells using a cDNA synthesis kit (Pharmacia). Next, PCR was carried out using said cDNA as a templete, and the oligomers S18 and A21 as primers, and using Ampli-Taq DNA polymerase (Takara). As a result, a DNA fragment of about 500 base pairs (bp) which is a fragment of GnT-V cDNA.

Next, further cDNA cloning was carried out using the cDNA thus obtained as a probe. Namely, this cDNA was used as a template and labeled with α32P-CTP using a random primer labeling kit (Amersham). The probe thus obtained was used to screen a human fetal liver cDNA library (Clone Tech?) according to a conventional procedure. Among the clones thus obtained, the longest clone was excised with EcoRI, and subcloned into pBluescript II ks(+) (Strate gene). Since the insert contains an EcoRI site, two fragments were obtained. Each of the two fragments was equenced, and B fragment of about 800 bp, and F fragment of about 1200 bp were obtained.

The sequences of the DNA fragments thus obtained were determined by Sequenase (U SB), and a result shown in SEQ ID NO: 8 was obtained. It was confirmed that this DNA fragment contains all the nucleotide sequences encoding five amino acid sequences obtained in Example 5.

Note, the cDNA fragment for GnT-V thus obtained does not reach to the stop codon at the 3'-terminus, and therefore 3'-terminal nucleotide sequence has not yet determined, but it is easy to determine the 3'-ter-

minal nucleotide sequence for a person with ordinary skill in the art.

Next, to express the GnT-V cDNA fragment in animal cells, the cDNA fragment was inserted into an expression vector. Namely, an expression vector pSVK3 (Pharmacia) was digested with EcoRI and SmaI so that the above-mentioned F fragment was excised at the EcoRI site and the SmaI site inside of the insert, and inserted into an expression vector (see, Fig. 9).

Next, the vector incorporating the F fragment was cleaved with EcoRI, and treated with CIP (calf intestinal phosphatase) to prevent self-ligation. Next, the B fragment excised at EcoRI sites was inserted into the above-treated expression vector to construct an expression vector containing both the F fragment and B fragment.

Example 9. Expression of cloned GnT-V cDNA and enzyme activity of the expressed protein

The expression vector constructed in Example 8 was used to express the GnT-V cDNA fragment in animal cells and GnT-V activity of the expressed protein was tested.

Expression vector containing GnT-V cDNA fragment was transfected to COS-1 cells, animal cells for expression, by electroporation. Namely, about 5×10^6 CO.S-1 cells previously prepared were suspended in HEPES buffer (50 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM $NaHPO_3$, 6 mM dextrose, pH 7.05), 60 μg of the above-mentioned plasmid was added thereon, and 800 μl of the suspension was subjected to electroporation in an electroporator (Bio-Rad) at 250V, 960 mF. Next, all the suspensions of COS-1 cells electroporated with GnT-V gene was plated in a peptri dish having the diameter of 6 cm, and cultured at 37°C in 5% CO_2 for 48 hours. The cultured cells were collected with a rubber-coated policeman bar, and suspended in 50 μl of PBS buffer, and the cells were disrupted with a sonicator. Next, the disruptant was centrifuged to collect a supernatant, and 15 μl of the supernatant was used to assay enzyme activity. A result is shown in Fig. 10.

From the result, it was confirmed that the cloned GnT-V cDNA fragment contain a minimum length necessary for expression of desired enzyme activity.

According to the present disclosure, UDP-N-acetylglucosamine: α-6-D-mannoside, β1,6-N-acetylglucosaminyl transferase was isolated and purified from a culture supernatant of QG cells derived from human lung cancer cells, cultured in a protein-free medium, and properties and partial amino acid structure thereof were determined. Moreover, the present inventors clarified substrate specificity to various sugar acceptors, using the purified enzyme. The sugar transfer enzyme having such specificity is an important enzyme responsible for control of sugar chain biosynthesis path way. Moreover, it is now being clarified that the present enzyme participates to modification of sugar chains accompanying malignant alteration of cells.

Considering the above, not only the present enzyme is useful as a maker for malignancy of cells, but also by establishing a screening system for specific inhibitors against the present enzyme, it becomes possible to design cancer-metastasis inhibitors. In addition, since β(1,6) branch structure can be introduced into various sugar acceptors exemplified using the present invention, oligosaccharides having β(1,6) branch structure can be industrially produced. Moreover, from an aspect of sugar chain technology, homogeneous formation of sugar chains in the production of desired substance by genetic engineering is possible by using a large amount of the present enzyme.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Suntory Limited
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(F) POSTAL CODE (ZIP):530

(A) NAME: Taniguchi, Naoyuki
(B) STREET: 201, 2-19-32 Uenohigashi, Toyonaka-shi
(C) CITY: Osaka
(E) COUNTRY: JAPAN
(F) POSTAL CODE (ZIP): none

(ii) TITLE OF INVENTION: N-acetylglucosaminyl transferase, gene coding therefor, corresponding vectors and transformed hosts, processes for production thereof

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WordPerfect 5.1

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER:EP 93306718.3

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: JP 4-245950
(B) FILING DATE: 24-AUG-1992

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: JP 5-237118
(B) FILING DATE: 06-AUG-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Thr Pro Trp Gly Lys
                  5
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Asn Ile Pro Ser Tyr Val
                  5
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
Val Leu Asp Ser Phe Gly Thr Glu Pro Glu Phe Asn His
                  5                  10
Ala Asn Tyr Ala
     15
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Asp Leu Gln Phe Leu Leu
                5
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Asn Thr Asp Phe Phe Ile Gly
                5
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
GGAATTC TTY AAY CAY GCN AAY TAY GC          27
        Phe Asn His Ala Asn Tyr Ala
                        5
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Synthetic DNA
(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
TTR TGN CTR AAR AAR TACTTAAGG                              24
Asn Thr Asp Phe Phe
                  5
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2095 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
CCGGCTGAAG CATCAGAATG GAAGTGAGGA AAGGCAACCA GCTGACACAG GAGCCAGAGT   60
GAGACCAGCA GACTCTCACA CTCAACCTAC ACCATGAATT TGTGTCTATC TTCTACGCGT  120
TAAGAGCCAA GGACAGGTGA AGTTGCCAGA GAGCA ATG GCT CTC TTC ACT CCG     173
                                       Met Ala Leu Phe Thr Pro
                                         1               5
TGG AAG TTG TCC TCT CAG AAG CTG GGC TTT TTC CTG GTG ACT TTT GGC    221
Trp Lys Leu Ser Ser Gln Lys Leu Gly Phe Phe Leu Val Thr Phe Gly
            10                  15                  20
TTC ATT TGG GGT ATG ATG CTT CTG CAC TTT ACC ATC CAG CAG CGA ACT    269
Phe Ile Trp Gly Met Met Leu Leu His Phe Thr Ile Gln Gln Arg Thr
        25                  30                  35
CAG CCT GAA AGC AGC TCC ATG CTG CGC GAG CAG ATC CTG GAC CTC AGC    317
Gln Pro Glu Ser Ser Ser Met Leu Arg Glu Gln Ile Leu Asp Leu Ser
    40                  45                  50
```

```
AAA AGG TAC ATC AAG GCA CTG GCA GAA GAA AAC AGG AAT GTG GTG GAT     365
Lys Arg Tyr Ile Lys Ala Leu Ala Glu Glu Asn Arg Asn Val Val Asp
 55                  60                  65                  70
GGG CCA TAC GCT GGA GTC ATG ACA GCT TAT GAT CTG AAG AAA ACC CTT     413
Gly Pro Tyr Ala Gly Val Met Thr Ala Tyr Asp Leu Lys Lys Thr Leu
                 75                  80                  85
GCT GTG TTA TTA GAT AAC ATT TTG CAG CGC ATT GGC AAG TTG GAG TCG     461
Ala Val Leu Leu Asp Asn Ile Leu Gln Arg Ile Gly Lys Leu Glu Ser
             90                  95                 100
AAG GTG GAC AAT CTT GTT GTC AAT GGC ACC GGA ACA AAC TCA ACC AAC     509
Lys Val Asp Asn Leu Val Val Asn Gly Thr Gly Thr Asn Ser Thr Asn
        105                 110                 115
TCC ACT ACA GCT GTT CCC AGC TTG GTT GCA CTT GAG AAA ATT AAT GTG     557
Ser Thr Thr Ala Val Pro Ser Leu Val Ala Leu Glu Lys Ile Asn Val
    120                 125                 130
GCA GAT ATC ATT AAC GGA GCT CAA GAA AAA TGT GTA TTG CCT CCT ATG     605
Ala Asp Ile Ile Asn Gly Ala Gln Glu Lys Cys Val Leu Pro Pro Met
135                 140                 145                 150
GAC GGC TAC CCT CAC TGT GAG GGA AAG ATC AAG TGG ATG AAA GAC ATG     653
Asp Gly Tyr Pro His Cys Glu Gly Lys Ile Lys Trp Met Lys Asp Met
                155                 160                 165
TGG CGT TCA GAT CCC TGC TAC GCA GAC TAT GGA GTG GAT GGA TCC ACC     701
Trp Arg Ser Asp Pro Cys Tyr Ala Asp Tyr Gly Val Asp Gly Ser Thr
            170                 175                 180
TGC TCT TTT TTT ATT TAC CTC AGT GAG GTT GAA AAT TGG TGT CCT CAT     749
Cys Ser Phe Phe Ile Tyr Leu Ser Glu Val Glu Asn Trp Cys Pro His
        185                 190                 195
TTA CCT TGG AGA GCA AAA AAT CCC TAC GAA GAA GCT GAT CAT AAT TCA     797
Leu Pro Trp Arg Ala Lys Asn Pro Tyr Glu Glu Ala Asp His Asn Ser
    200                 205                 210
TTG GCG GAA ATT CGT ACA GAT TTT AAT ATT CTC TAC AGT ATG ATG AAA     845
Leu Ala Glu Ile Arg Thr Asp Phe Asn Ile Leu Tyr Ser Met Met Lys
215                 220                 225                 230
AAG CAT GAA GAA TTC CGG TGG ATG AGA CTA CGG ATC CGG CGA ATG GCT     893
Lys His Glu Glu Phe Arg Trp Met Arg Leu Arg Ile Arg Arg Met Ala
                235                 240                 245
GAC GCA TGG ATC CAA GCA ATC AAG TCC CTG GCA GAA AAG CAG AAC CTT     941
Asp Ala Trp Ile Gln Ala Ile Lys Ser Leu Ala Glu Lys Gln Asn Leu
            250                 255                 260
GAA AAG AGA AAG CGG AAG AAA GTC CTC GTT CAC CTG GGA CTC CTG ACC     989
Glu Lys Arg Lys Arg Lys Lys Val Leu Val His Leu Gly Leu Leu Thr
        265                 270                 275
AAG GAA TCT GGA TTT AAG ATT GCA GAG ACA GCT TTC AGT GGT GGC CCT    1037
Lys Glu Ser Gly Phe Lys Ile Ala Glu Thr Ala Phe Ser Gly Gly Pro
    280                 285                 290
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CTT GGT GAA TTA GTT CAA TGG AGT GAT TTA ATT ACA TCT CTG TAC TTA    1085
Leu Gly Glu Leu Val Gln Trp Ser Asp Leu Ile Thr Ser Leu Tyr Leu
295                 300                 305                 310
CTG GGC CAT GAC ATT AGG ATT TCA GCT TCA CTG GCT GAG CTC AAG GAA    1133
Leu Gly His Asp Ile Arg Ile Ser Ala Ser Leu Ala Glu Leu Lys Glu
            315                 320                 325
ATC ATG AAG AAG GTT GTA GGA AAC CGA TCT GGC TGC CCA ACT GTA GGA    1181
Ile Met Lys Lys Val Val Gly Asn Arg Ser Gly Cys Pro Thr Val Gly
        330                 335                 340
GAC AGA ATT GTT GAG CTC ATT TAC ATT GAT ATT GTA GGA CTT GCT CAA    1229
Asp Arg Ile Val Glu Leu Ile Tyr Ile Asp Ile Val Gly Leu Ala Gln
    345                 350                 355
TTC AAG AAA ACT CTT GGA CCA TCC TGG GTT CAT TAC CAG TGC ATG CTC    1277
Phe Lys Lys Thr Leu Gly Pro Ser Trp Val His Tyr Gln Cys Met Leu
    360                 365                 375
CGA GTC CTT GAT TCA TTT GGT ACT GAA CCC GAA TTT AAT CAT GCA AAT    1325
Arg Val Leu Asp Ser Phe Gly Thr Glu Pro Glu Phe Asn His Ala Asn
380                 385                 390                 395
TAT GCC CAA TCG AAA GGC CAC AAG ACC CCT TGG GGA AAA TGG AAT CTG    1373
Tyr Ala Gln Ser Lys Gly His Lys Thr Pro Trp Gly Lys Trp Asn Leu
            400                 405                 410
AAC CCT CAG CAG TTT TAT ACC ATG TTC CCT CAT ACC CCA GAC AAC AGC    1421
Asn Pro Gln Gln Phe Tyr Thr Met Phe Pro His Thr Pro Asp Asn Ser
        415                 420                 425
TTT CTG GGG TTT GTG GTT GAG CAG CAC CTG AAC TCC AGT GAT ATC CAC    1469
Phe Leu Gly Phe Val Val Glu Gln His Leu Asn Ser Ser Asp Ile His
    430                 435                 440
CAC ATT AAT GAA ATC AAA AGG CAG AAC CAG TCC CTT GTG TAT GGC AAA    1517
His Ile Asn Glu Ile Lys Arg Gln Asn Gln Ser Leu Val Tyr Gly Lys
    445                 450                 455
GTG GAT AGC TTC TGG AAG AAT AAG AAG ATC TAC TTG GAC ATT ATT CAC    1565
Val Asp Ser Phe Trp Lys Asn Lys Lys Ile Tyr Leu Asp Ile Ile His
460                 465                 470                 475
ACA TAC ATG GAA GTG CAT GCA ACT GTT TAT GGC TCC AGC ACA AAG AAT    1613
Thr Tyr Met Glu Val His Ala Thr Val Tyr Gly Ser Ser Thr Lys Asn
            480                 485                 490
ATT CCC AGT TAC GTG AAA AAC CAT GGT ATC CTC AGT GGA CGG GAC CTG    1661
Ile Pro Ser Tyr Val Lys Asn His Gly Ile Leu Ser Gly Arg Asp Leu
        495                 500                 505
CAG TTC CTT CTT CGA GAA ACC AAG TTG TTT GTT GGA CTT GGG TTC CCT    1709
Gln Phe Leu Leu Arg Glu Thr Lys Leu Phe Val Gly Leu Gly Phe Pro
    510                 515                 520
TAC GAG GGC CCA GCT CCC CTG GAA GCT ATC GCA AAT GGA TGT GCT TTT    1757
Tyr Glu Gly Pro Ala Pro Leu Glu Ala Ile Ala Asn Gly Cys Ala Phe
    525                 530                 535
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CTG AAT CCC AAG TTC AAC CCA CCC AAA AGC AGC AAA AAC ACA GAC TTT   1805
Leu Asn Pro Lys Phe Asn Pro Pro Lys Ser Ser Lys Asn Thr Asp Phe
540                 545                 550                 555
TTC ATT GGC AAG CCA ACT CTG AGA GAG CTG ACA TCC CAG CAT CCT TAC   1853
Phe Ile Gly Lys Pro Thr Leu Arg Glu Leu Thr Ser Gln His Pro Tyr
                560                 565                 570
GCT GAA GTT TTC ATC GGG CGG CCA CAT GTG TGG ACT GTT GAC CTC AAC   1901
Ala Glu Val Phe Ile Gly Arg Pro His Val Trp Thr Val Asp Leu Asn
            575                 580                 585
AAT CAG GAG GAA GTA GAG GAT GCA GTG AAA GCA ATT TTA AAT CAG AAG   1949
Asn Gln Glu Glu Val Glu Asp Ala Val Lys Ala Ile Leu Asn Gln Lys
        590                 595                 600
ATT GAG CCA TAC ATG CCA TAT GAA TTT ACG TGC GAG GGG ATG CTA CAG   1997
Ile Glu Pro Tyr Met Pro Tyr Glu Phe Thr Cys Glu Gly Met Leu Gln
    605                 610                 615
AGA ATC AAT GCT TTC ATT GAA AAA CAG GAC TTC TGC CAT GGG CAA GTG   2045
Arg Ile Asn Ala Phe Ile Glu Lys Gln Asp Phe Cys His Gly Gln Val
620                 625                 630                 635
ATG TGG CCA CCC CTC AGC GCC CTA CAG GTC AAG CTT GCT GAG CCC GGG   2093
Met Trp Pro Pro Leu Ser Ala Leu Gln Val Lys Leu Ala Glu Pro Gly
                640                 645                 650
CC                                                                2095
```

Claims

1. A β1,6-N-acetylglucosaminyl transferase having the following properties:
   (1) Action: it transfers N-acetylglucosamine from UDP-N-acetylglucosamine to α-6-D-mannoside;
   (2) Substrate specificity: it shows a reactivity of about 79% for GnGnF-bi-PA, about 125% for GnGnGn-tri-PA and about 66% for GnM-PA, when taking a reactivity for GnGn-bi-PA as 100%;
   (3) Optimum pH: 6.2 to 6.3;
   (4) Inhibition, Activation and Stability: Mn^{2+} is not necessary for expression of activity, and the activity is not inhibited in the presence of 20 mM EDTA;
   (5) Molecular weight: about 73,000 as determined by SDS-PAGE in the absence of reducing agent; and about 73,000 and about 60,000 as determined in the presence of a reducing agent;
   (6) Km value: 133 μM and 3.5 mM for acceptor GnGn-bi-PA and donor UDP-GlcNAc, respectively; and
   (7) It includes the following peptide fragments:
      (1) Thr-Pro-Trp-Gly-Lys
      (2) Asn-Ile-Pro-Ser-Tyr-Val
      (3) Val-Leu-Asp-Ser-Phe-Gly-Thr-Glu-Pro-Glu-Phe-Asn-His-Ala-Asn-Tyr-Ala
      (4) Asp-Leu-Gln-Phe-Leu-Leu
      (5) Asn-Thr-Asp-Phe-Phe-Ile-Gly.

2. A β1,6-N-acetylglucosaminyl transferase consisting essentially of an amino acid sequence shown in SEQ ID No: 8.

3. A β1,6-N-acetylglucosaminyl transferase consisting essentially of an amino acid sequence having one or more amino acid modifications in the amino acid sequence shown in SEQ ID No: 8.

4. A β1,6-N-acetylglucosaminyl transferase according to claim 3 wherein the amino acid modification is addition, deletion, replacement with other amino acids, or a combination thereof.

5. DNA coding for β1,6-N-acetylglucosaminyl transferase according to any one of claims 1 to 4.

6. DNA according to claim 5 consisting essentially of a nucleotide sequence as shown in SEQ ID No. 8.

7. An expression vector comprising DNA according to claim 5 or claim 6.

8. A host transformed with an expression vector according to claim 7.

9. A process for production of a β1,6-N-acetylglucosaminyl transferase according to any one of claims 1 to 4, comprising the steps of:
   culturing or raising a host transformed with a DNA coding for said enzyme, and
   recovering the enzyme from the culture or the raised host.

Fig. 1

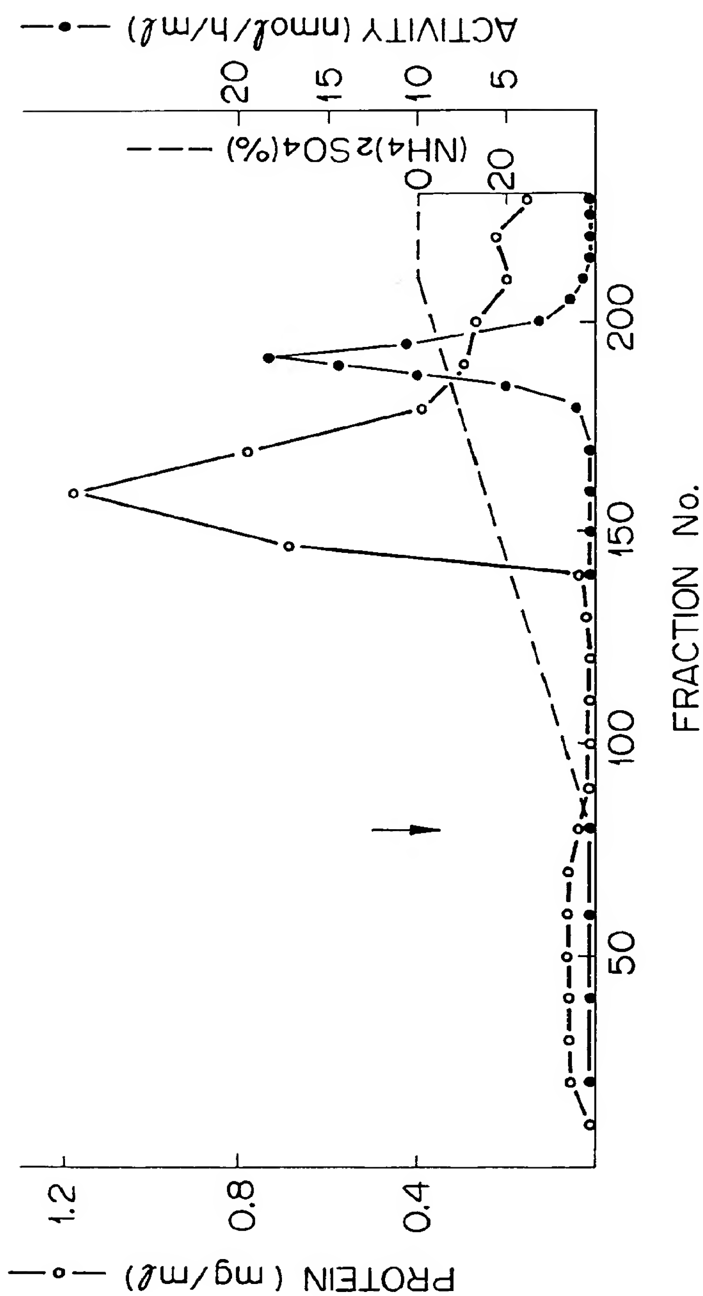

ACTIVITY (nmol/h/ml)
20
15
10
5
(NH4)2SO4(%)
0
20
PROTEIN (mg/ml)
1.2
0.8
0.4
50
100
150
200
FRACTION No.

Fig. 2

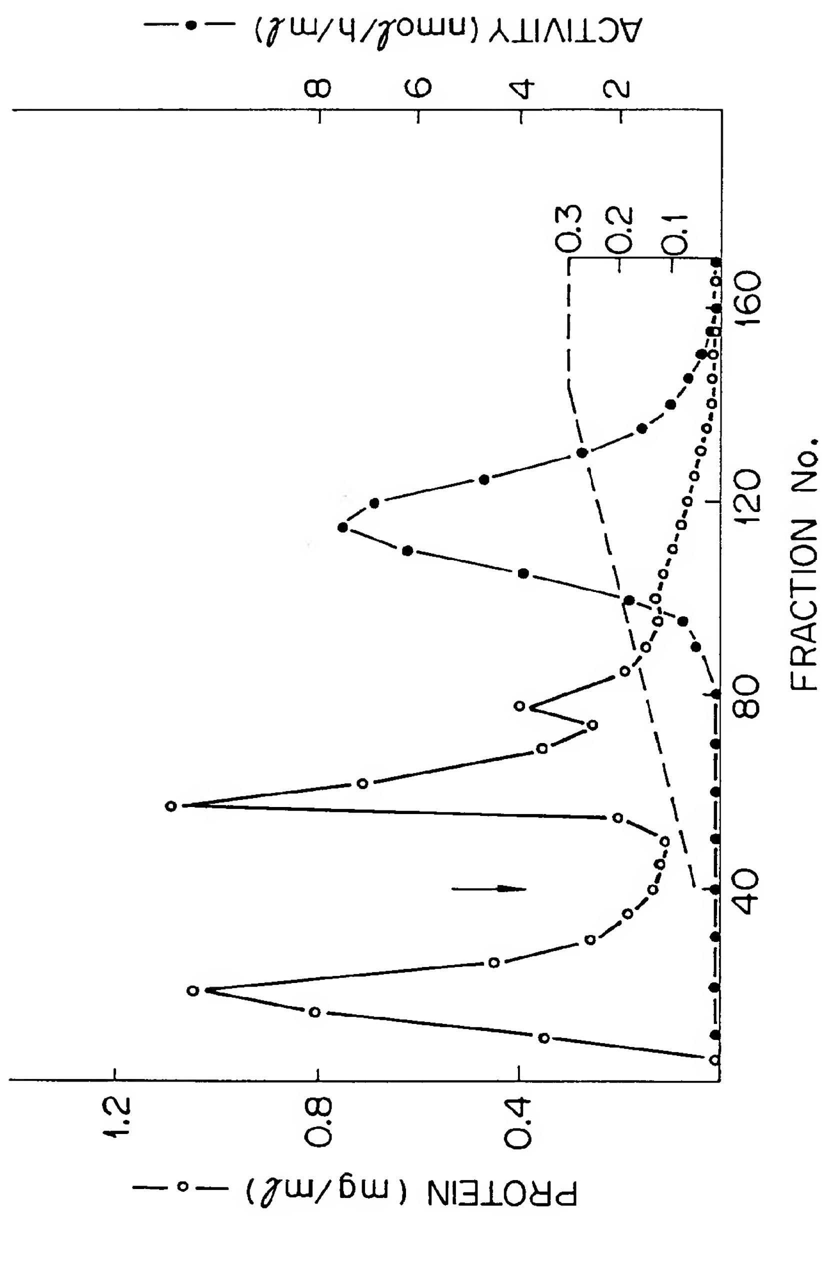

PROTEIN (mg/mℓ) —○—
1.2
0.8
0.4
ACTIVITY (nmoℓ/h/mℓ) —●—
8
6
4
2
0.3
0.2
0.1
40
80
120
160
FRACTION No.

Fig. 3

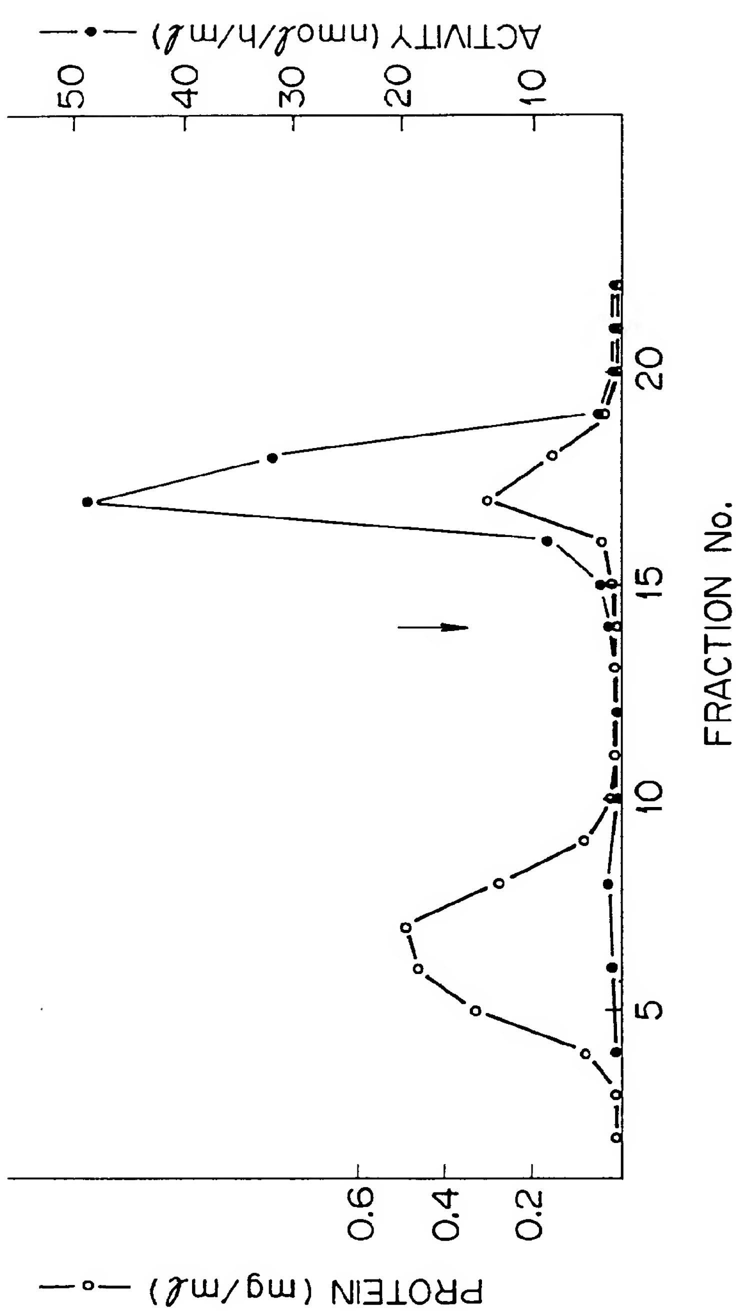
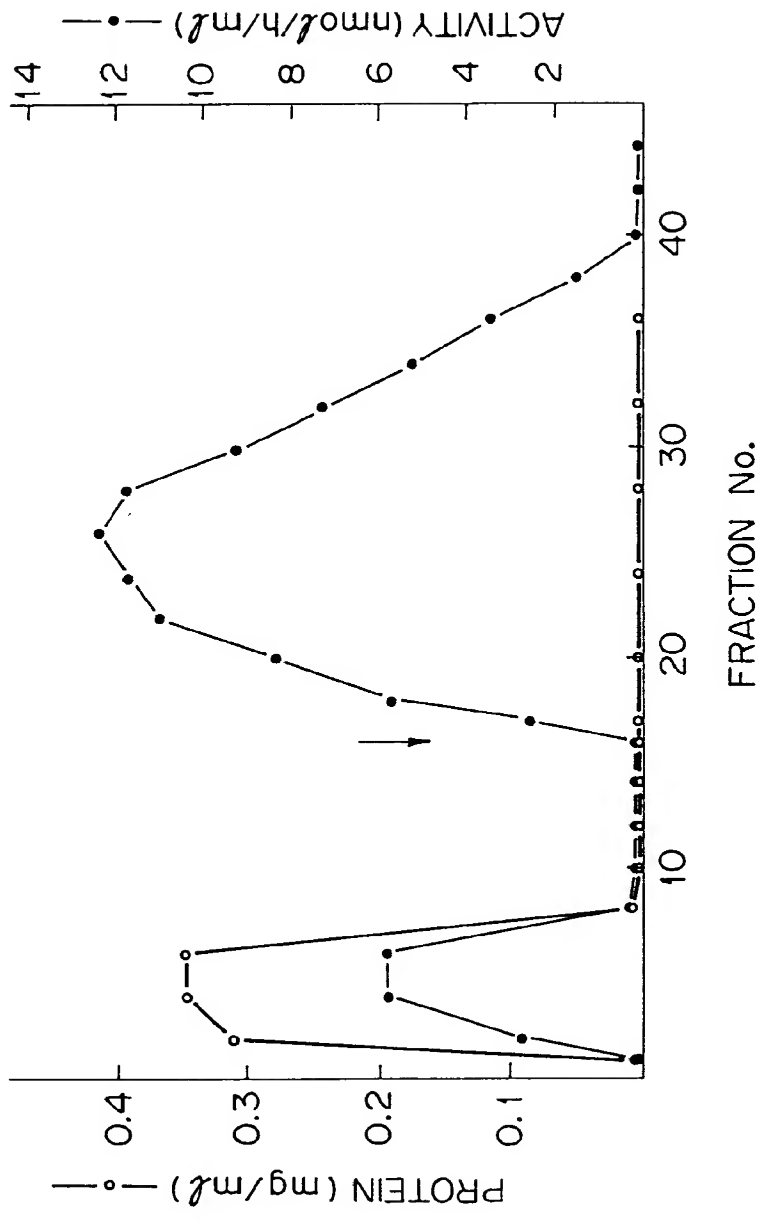

ACTIVITY (nmoℓ/h/mℓ)
50
40
30
20
10
PROTEIN (mg/mℓ)
0.6
0.4
0.2
5
10
15
20
FRACTION No.

Fig. 4


ACTIVITY (nmoℓ/h/mℓ)
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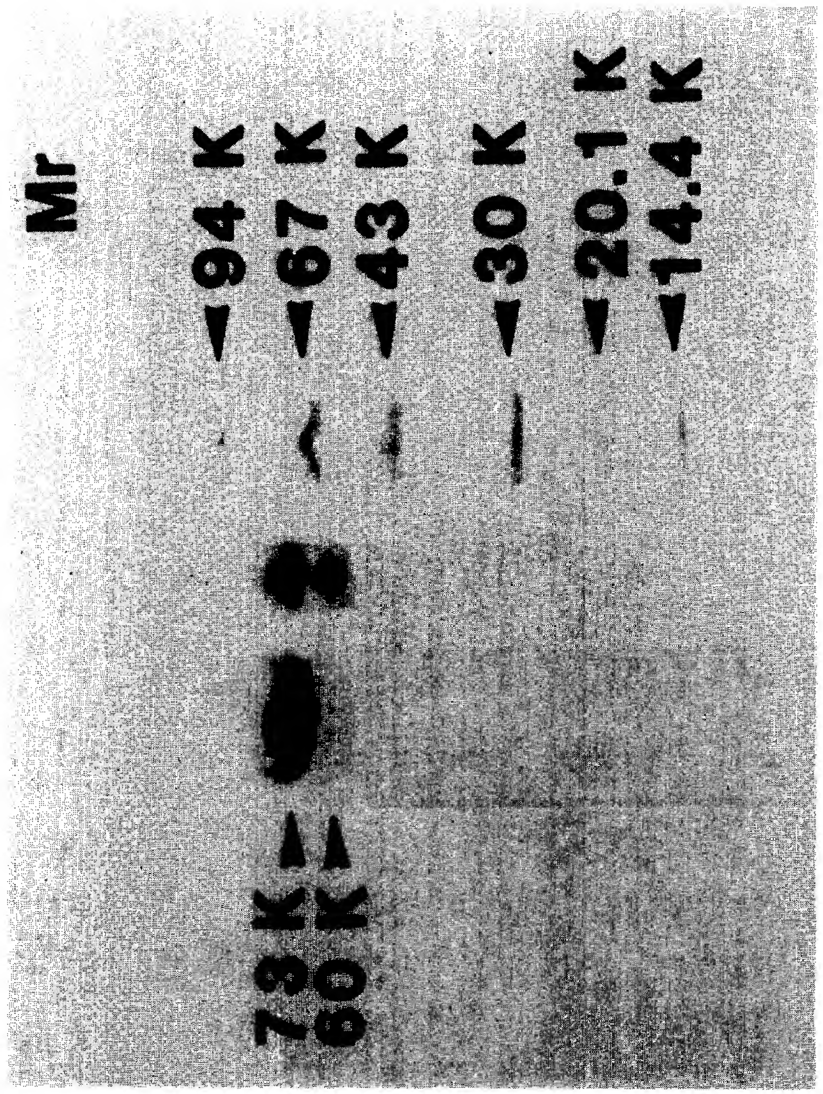
12
10
8
6
4
2
PROTEIN (mg/mℓ)
0.4
0.3
0.2
0.1
10
20
30
40
FRACTION No.

Fig. 5



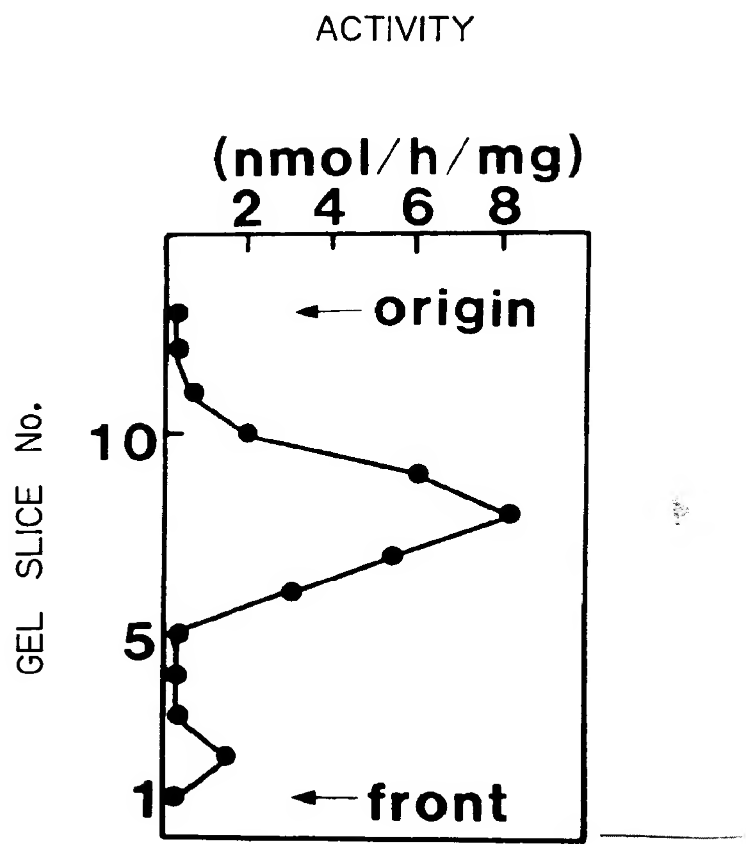

ACTIVITY
(nmol/h/mg)
2 4 6 8
origin
front
GEL SLICE No.
10
5
1

Fig. 7

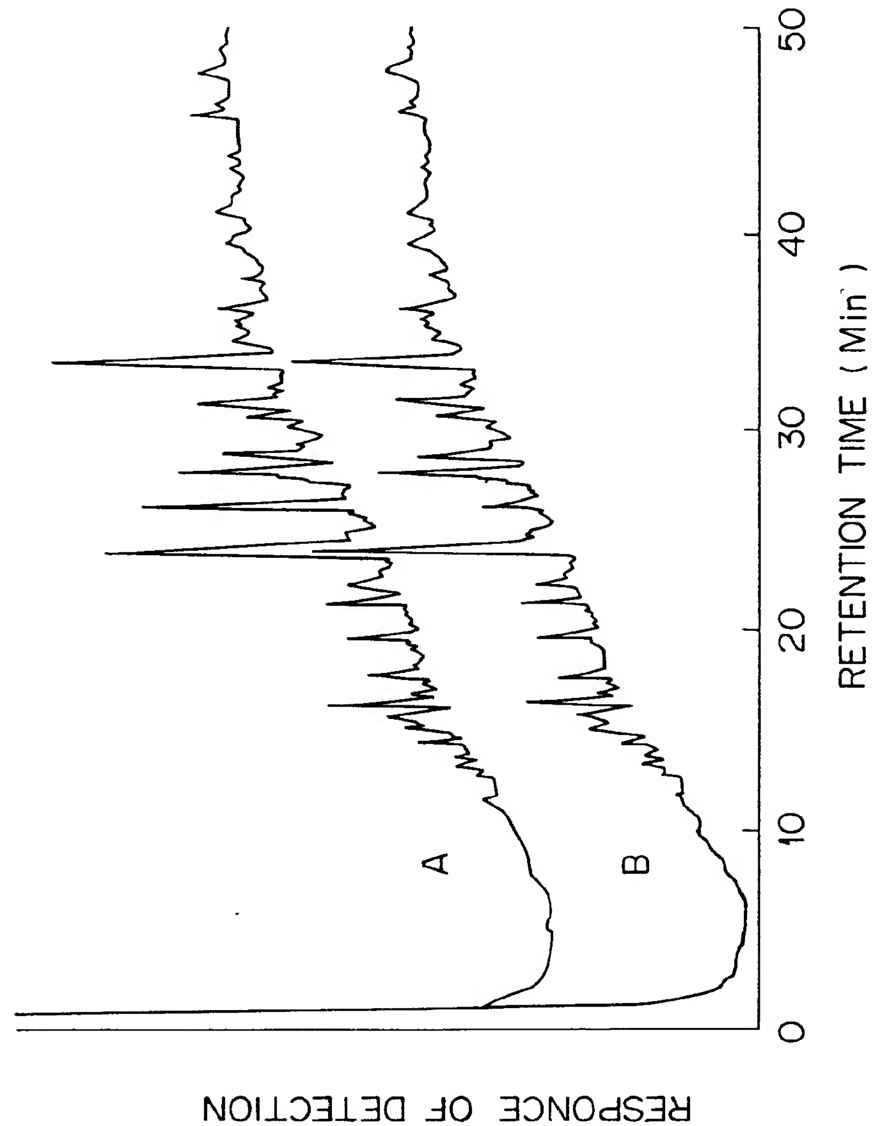
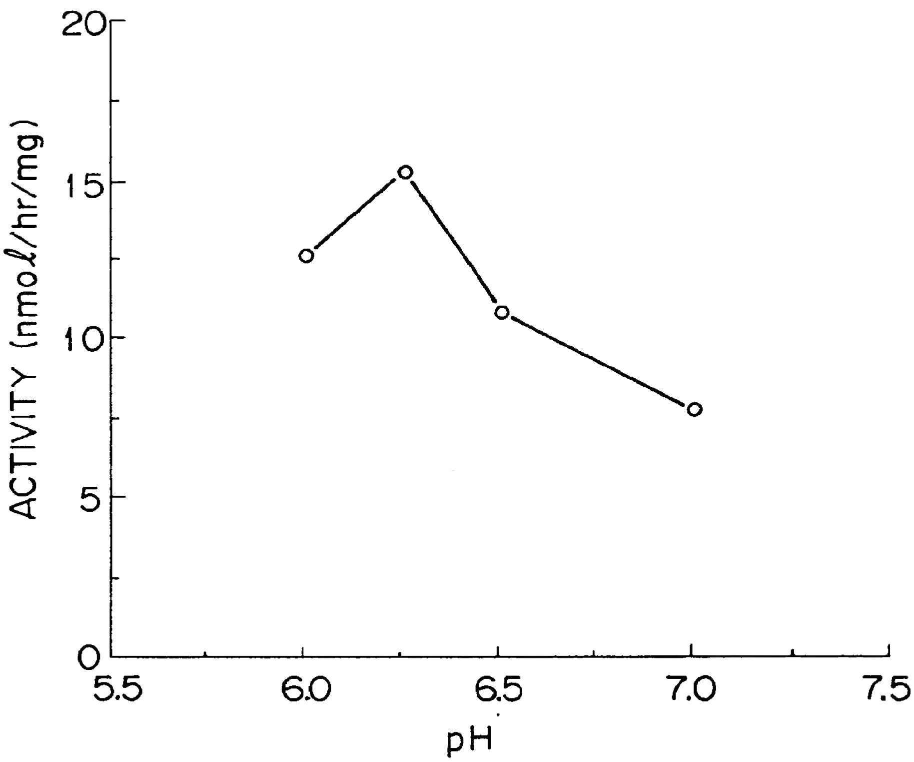


Fig. 8



pH	ACTIVITY
6.0	12.7 (nmol/hr/mg)
6.25	15.3
6.5	10.8
7.0	7.8

Fig. 9

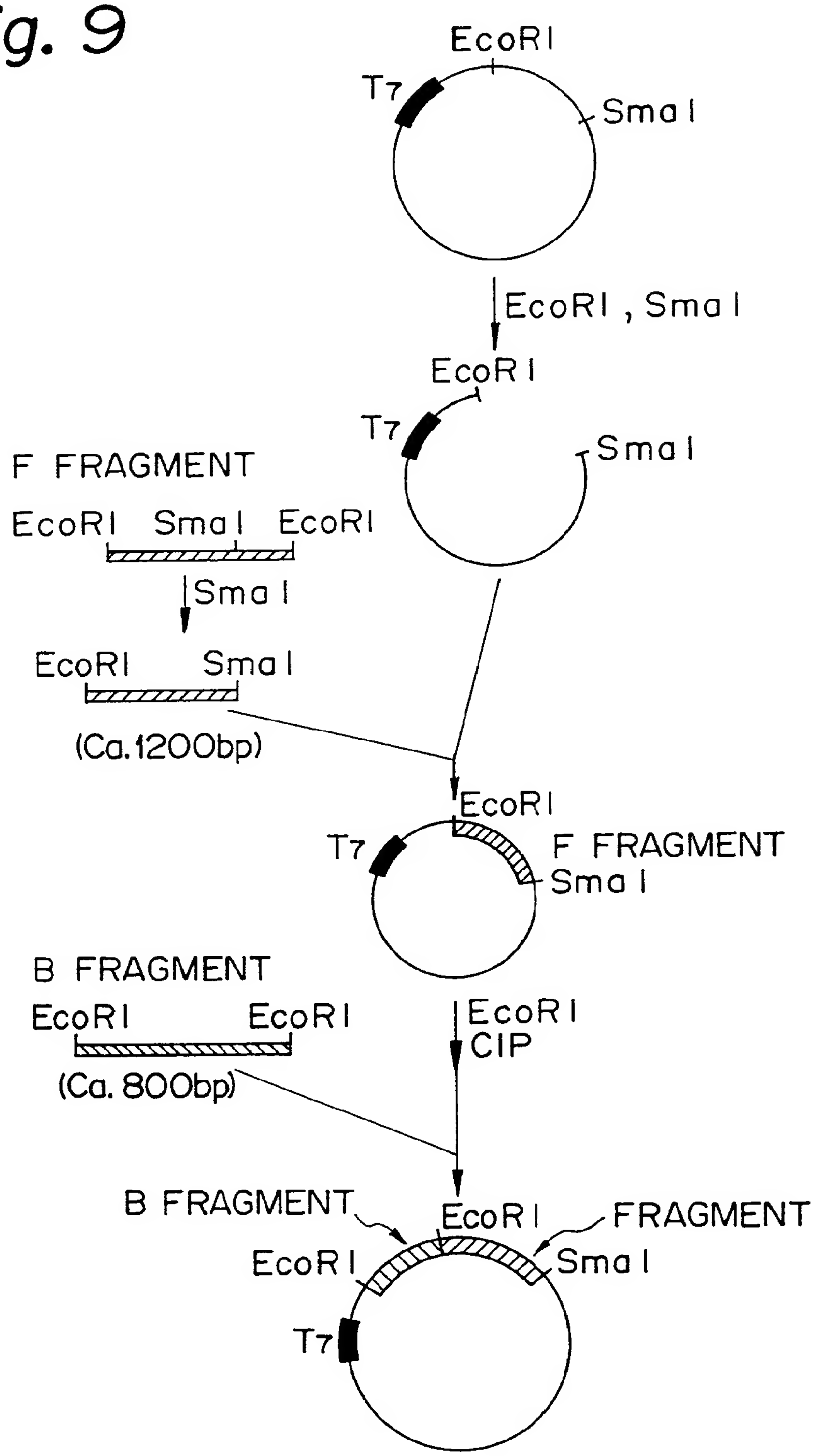

EcoRI
T7
Sma I
EcoRI , Sma I
EcoR I
T7
Sma I
F FRAGMENT
EcoRI Sma I EcoRI
Sma I
EcoRI Sma I
(Ca.1200bp)
EcoRI
T7
F FRAGMENT
Sma I
B FRAGMENT
EcoRI EcoR I
EcoR I
CIP
(Ca. 800bp)
B FRAGMENT
EcoR I
FRAGMENT
EcoR I
Sma I
T7

Fig. 10